STAPHYLOCOCCAL VIROLYSIN, A PHAGE-INDUCED LYSIN

Its Differentiation from the Autolysin of Normal Cells*

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SUMMARY

Virolysin is a lysin which appears in *Staphylococcus aureus* K_1 cells infected with phage P_{14}; together with phage, virolysin is released from phage-infected cells at the time of lysis. Autolysin is a lysin formed by uninfected cells of the K_1 strain; autolysin is released from uninfected cells by autolysis.

They show the following similarities: Both agents act within the genus *Micrococcus*. They lyse cells only after the cell has been subjected to a damaging or "sensitizing" treatment, such as heat, bacteriophage, acetone, or ultraviolet irradiation. The course of lysis of heated cells by both lysins has been found to proceed in a similar manner. A constant percentage of cells is lysed, independent of the concentration of lysin; the residual cells remain resistant to either lysin. Lysis proceeds logarithmically with time, and the velocity constants K are proportional to the lysin concentration. K increases with increasing temperature.

Both lysins are unaffected by antiserum to the phage. They are inhibited alike by a number of chemicals, including known enzyme inhibitors. Both agents are destroyed by proteolytic enzymes and are precipitated by 40 per cent saturation with $(NH_4)_2SO_4$. Both lysins are very thermolabile.

The two lysins differ with respect to their pH optimum, antigenic relationship and specificity for *Micrococcus lysodeikticus*.

These results suggest that (1) both lysins have many properties associated with enzymes, (2) the lysis of heated cells, which they produce, has some of the characteristics of a chemical reaction, (3) the lysin from the phage-infected cell is clearly different from the lysin of the uninfected cell.

INTRODUCTION

A lysin has been detected in the supernatant layer of ultracentrifuged phage lysates of *S. aureus* K_1 and 145 (Ralston, Baer, Lieberman, and Krueger, 1955).

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The name virolysin has been proposed to designate those lysins which appear as a result of virus infection and are not detectable in uninfected host cells.

A number of authors have proposed that the autolytic systems of the host are involved in the lyses that result from various phage-host interactions (Bronfenbrenner and Muckenfuss, 1927; Wollman and Wollman, 1933; Huppert and Panijel, 1954; Puck, 1953). This paper is concerned chiefly with the relationship between the virolysin of phage lysates and the autolysin of normal host cells. The problems of the intracellular appearance of virolysin, its function in lysis-from-without and in the lysis of infected cells at the end of the latent period are described elsewhere (Ralston *et al.*, data to be published, 1957 *a*, *b*).

Materials and Methods

Host-Virus Systems.—These experiments have been performed with phages P_{14} and P_1 and hosts *S. aureus* K_1 and 145. The systems and the method of phage assay have been described (Jones and Krueger, 1951; Ralston and Krueger, 1952, 1954).

Media.—(1) Tryptose phosphate broth, Difco, 30 gm. per liter, (2) double strength tryptose phosphate broth, 60 gm. per liter, (3) tryptose phosphate agar, 30 gm. tryptose phosphate and 25 gm. agar per liter, (4) mineral salts broth, M/5000 Mn^{++}, Mg^{++}, and Ca^{++}, M/15 KH_2/K_2HPO_4, 0.14 M NaCl, and 3 gm. tryptose phosphate per liter.

Preparation of Heat-Killed Cells.—Cells were grown on tryptose phosphate agar at 37°C. Each plate was inoculated with 1×10^9 bacteria per ml. from a 16 to 18 hour slant of *S. aureus* K_1 (unless otherwise stated). Logarithmic phase cells were collected after 3 to 4 hours; resting phase cells after 16 to 24 hours. They were both harvested in 0.85 per cent saline, usually washed once, resuspended in saline to *ca.* 5×10^{10} cells per ml., and were killed by heating at 56–100°C. for over 30 minutes. Suspensions were then stored at 4°C.

Lysis Measurements.—Lytic activity was measured by turbidimetric determination of cellular lysis, using a Klett-Summerson photoelectric colorimeter. For example, 5 ml. of a suitable dilution of the lysin were mixed with a small volume of bacterial cells so that the mixture contained a known initial concentration, generally 6 to 10×10^8 bacteria per ml. For each test, all samples received a constant, initial amount of cells (unless otherwise stated). The samples were incubated at 37°C. and readings were made at intervals. The changes in optical density were converted to changes in bacterial count by the use of standard curves prepared from direct microscopic counts of *S. aureus* K_1. The amount of bacterial debris remaining after lysis did not interfere with the estimates of cells lysed.

From the data, the course of lysis could be represented by plotting the logarithm of the unlysed bacteria against time at any given temperature, etc.

Estimates of lysin activity were based upon determinations of the initial velocity constants, K, calculated from the equation

$$K = \frac{2.3}{t} \log \frac{B_o}{B_o - B_t},$$

in which B_o represents the initial concentration of cells per ml., B_t are the cells per milliliter lysed in any given time interval, t. These "constants" are reported only for purposes of comparison within an individual experiment.

Less accurate estimates of lysin activity were made by determining the per cent bacteria lysed in a short time interval, and comparing the per cent lysis instead of the velocity constants, K, for different samples.

Procedure and Results

It has been found that neither virolysin nor autolysin affects freshly harvested, undamaged cells; both lysins, however, cause lysis after cells have been "sensitized" by a variety of damaging treatments: heat (from 45–120°C.), acetone, detergents, ultraviolet irradiation, penicillin. Phage also causes sensitization, and a combination of phage and lysin added to living cells initiates a lysis resembling typical lysis-from-without (Ralston *et al.*, 1955).

In this paper, the comparative study of the two lysins has been based mainly on their action on cells killed by heat. Identical results were obtained with cells killed at 56°, 100°C., or autoclaved for 20 minutes at 15 pounds per square inch. This method of sensitization was used to destroy intracellular enzymes. Unless otherwise noted, the experiments were performed with the 14 (K_1) virolysin and the K_1 autolysin.

I. Source of Virolysin and Autolysin

A. Preparation of Virolysin.—Virolysin is obtained from lysates of phage-infected cells. Phage lysates are prepared by inoculating double strength tryptose-phosphate broth with an 18 hour agar-grown *S. aureus* K_1 suspension at a concentration of 1×10^8 cells per ml. and phage 14 at 1×10^6 particles per ml. The cell-phage mixture is shaken at 37°C. until lysis occurs (usually 3 hours). The lysate contains both virolysin and phage (*ca.* 10^{10} particles per ml.).

This method of virolysin preparation involves several rounds of infection, since the cells outnumber the phage particles by 100 to 1. Fig. 1 shows the appearance of lysin and phage, as well as the changes in bacterial numbers during the process. Appreciable quantities of virolysin and phage appear at the time of mass lysis.

Lysates containing virolysin can also be obtained from cell suspensions undergoing a single cycle of infection, by adding 3 to 5 particles per cell to 1×10^8 bacteria per ml. under conditions similar to those described above. Lysis occurs at the end of a 40 minute latent period, and the release of virolysin and phage from the cell coincides with lysis. The appearance and increase in virolysin inside the infected cell during the latent period are described elsewhere (Ralston *et al.*, 1955; Ralston *et al.*, 1957 *b*).

Virolysin is separated from the heavier phage particles in the lysates by

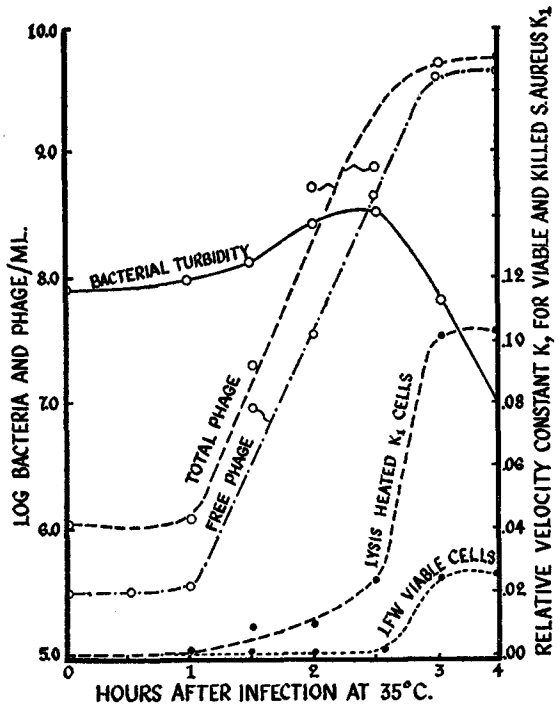

FIG. 1. Production of phage and virolysin in double strength tryptose phosphate broth at 37°C., when the initial phage/bacterium ratio was 1/100. The cell-phage mixture was removed at intervals and chilled in an ice bath. Total and free phage was determined by plaque counts of super-cel filtered and unfiltered samples. Virolysin activity was determined by lysis tests of samples which had been centrifuged to spin out the residual cells. Undiluted samples of the supernatant layer were mixed with 8×10^8 heat-killed *S. aureus* K_1 cells per ml. and incubated at 37°C. The velocity constant, K per minute, was calculated from the turbidimetric measurements of bacterial lysis. Undiluted samples of the supernatant layer were also mixed with 8×10^8 living K_1 cells per ml. and the velocity constant, K per minute, was calculated for their lysis at 37°C., as represented by the curve marked *LFW*. The lysis of living cells depends upon the presence of sufficient quantities of free phage to sensitize and of virolysin to lyse the cells. This lysis is considered to be a case of "lysis-from-without" (*LFW*).

spinning the lysate at 30,000 × *g* for 45 minutes at 6°C. in a Spinco model L ultracentrifuge. After 2 to 3 cycles of centrifugation, the phage in the supernate is reduced to 10^6 to 10^7 particles per ml. The lysin in the supernate can be precipitated by 40 per cent saturation with $(NH_4)_2SO_4$ at 4°C. The precipitate is then resuspended in mineral salts broth.

Virolysin can also be prepared by infecting the K_1 strain with phage 1 or the 145 strain of *S. aureus* with phage 1 or 14.

B. Preparation of Autolysin.—Autolysin is prepared from autolysates of uninfected cells of the K_1 strain grown in tryptose phosphate broth. Cells, grown on tryptose phosphate agar for 18 hours at 37°C., are inoculated into broth to a concentration of 1×10^8 bacteria per ml. and shaken at 37°C. for 6 hours. There is an increase in cell numbers, beginning at the 1st hour, from 1.0×10^8 cells per ml. to 2.5×10^9 cells per ml. at the 4th hour. The cell numbers then remain unchanged. At the 6th hour, the culture is removed from the shaker and stored at 4°C. for 1 to 5 days. During the storage period, autolysis occurs and, with it, the release of active autolysin.

Autolysis occurs much more rapidly with 3 hour cells stored at 4°C. or with 6 hour cells, layered with toluene and allowed to remain at 37°C.; however, under these conditions, either no autolysin is released, or, if present, it is very unstable. Agar-grown cultures autolyze very poorly and release little autolysin.

The autolysates are very viscous. Centrifugation of the autolysin at 30,000 $\times$ *g* for 45 minutes at 6°C. in the model L Spinco ultracentrifuge separates the lysin in the supernatant layer from the viscous material in the pellet. The lysin in the supernate can be precipitated with $(NH_4)_2SO_4$ at 40 per cent saturation at 4°C., and the precipitate resuspended in mineral salts broth.

C. "Crude" and "Semipurified" Lysins.—The ultracentrifuged supernates of the phage lysate or autolysate are referred to as "crude" lysins. The resuspended material from $(NH_4)_2SO_4$ precipitates is called "semipurified" lysin. Unless the term semipurified lysin is used, the experimental findings presented apply to the crude lysin.

Both lysins are adsorbed by a number of materials, including super-cel, alumina, charcoal; both are destroyed by the proteolytic enzymes, trypsin, chymotrypsin, and papain, but are not affected by ribonuclease or desoxyribonuclease.

II. Quantitative Aspects of the Lysis of Heat-Killed Cells by Autolysin and Virolysin

A. Effect of Cell (Substrate) Concentration.—Constant amounts of each crude lysin were mixed with varying amounts of cells. With both lysins, the curves representing the logarithms of the surviving bacteria per milliliter plotted against time, for each substrate concentration, showed an initial rapid decrease, followed by a slower lysis and an eventual end-point in the presence of considerable numbers (*ca.* 50 per cent) of unlysed cells. Microscopic study has shown the residual cells to be Gram-negative. They are resistant to fresh additions of either lysin and also to lysozyme, but are susceptible to trypsin.

When the data are corrected for these resistant cells, the course of lysis is

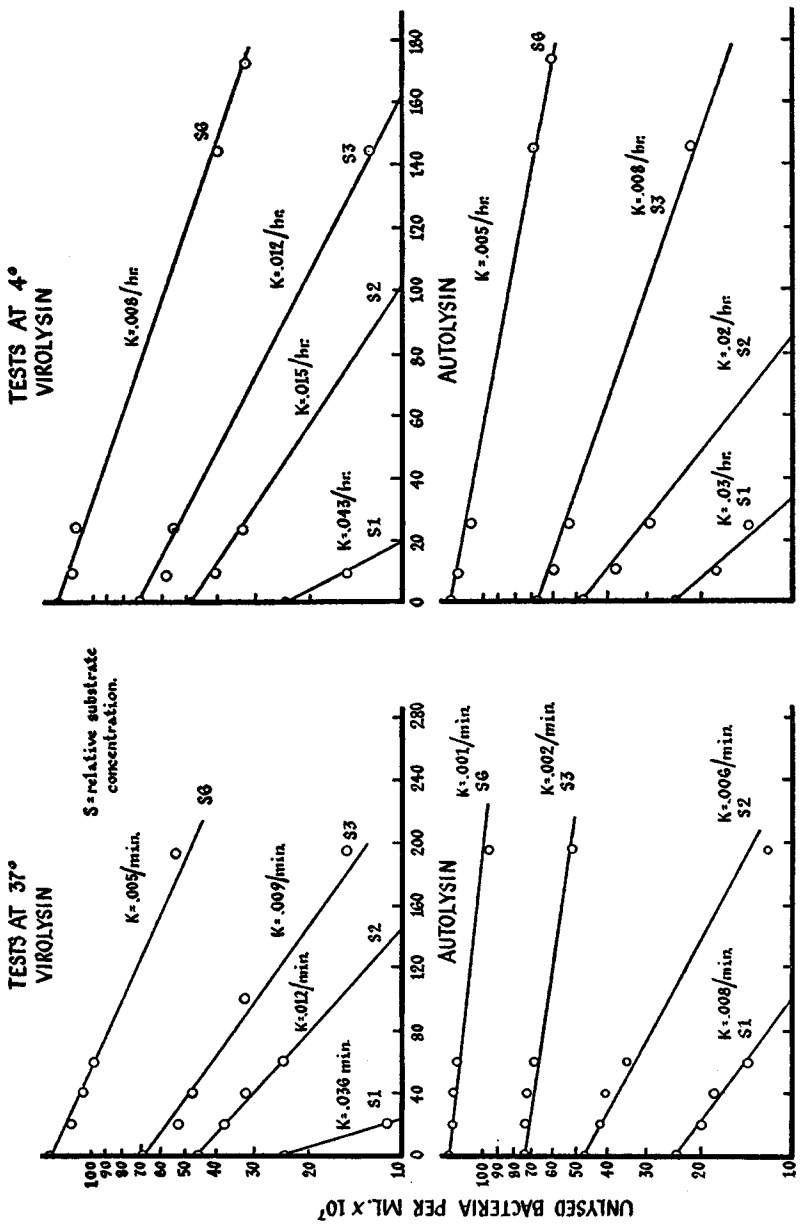

FIG. 2. Effect of substrate concentration (heat-killed K_1 cells) on lysis by constant amounts of autolysin and virolysin at their pH optima. (Data corrected for lysis-resistant cells.)

found to proceed logarithmically. With both lysins, the velocity constants, K, calculated from these curves, are decreased by raising the initial substrate concentration (Fig. 2).

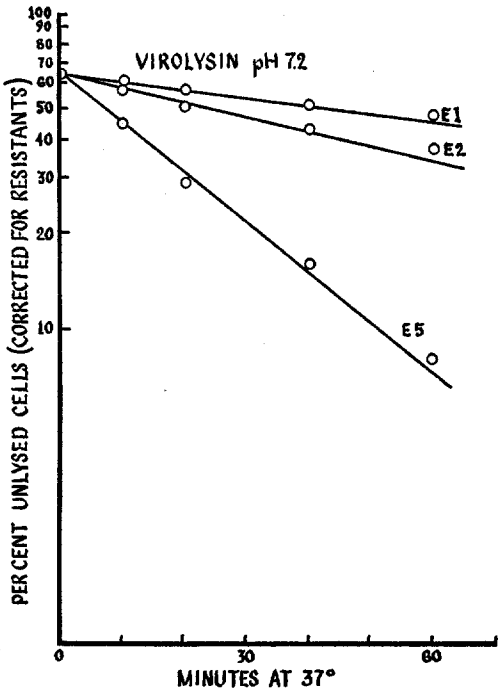

FIG. 3. Effect of virolysin concentration on lysis of constant amounts of heated K_1 cells at 37°C. Initial cells = 7×10^8/ml.

Relative lysin concentration (E)	Velocity constant (K)	$\frac{(K)}{(E)}$
1	0.005	0.005
2	0.010	0.005
5	0.038	0.007

B. Effect of Lysin Concentration.—Varying dilutions of each crude lysin were mixed with constant numbers of heat-killed cells at the pH optimum of each lysin. At all concentrations of the lysin, lysis came to an end-point in the presence of a constant number (*ca.* 50 per cent) of unlysed cells. Velocity constants, K, calculated from these curves (corrected for the resistant cells) showed a direct relationship to lysin concentration (Figs. 3 and 4).

C. Effect of Temperature.—Constant amounts of each crude lysin were added to constant amounts of cells at the pH optimum of each lysin and at tem-

peratures of 0, 28, and 37°C. With both lysins, lysis proceeds in the cold; the rate increases with rise in temperature. Velocity constants were calculated for the straight line portion of each curve, and these figures were used for deter-

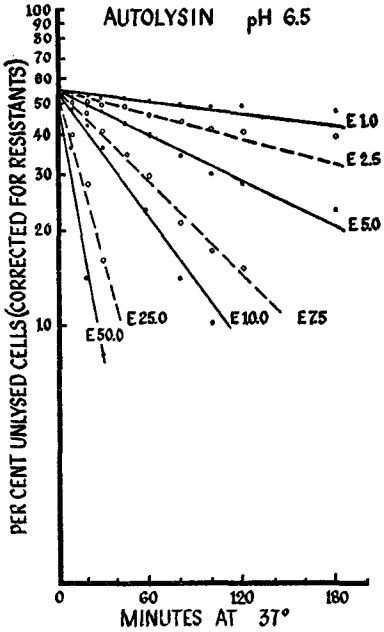

FIG. 4. Effect of autolysin concentration on lysis of constant amounts of heated K_1 cells at 37°C. Initial cells = 8×10^8/ml.

Relative lysin concentration (E)	Velocity constant (K/min.)	$\frac{(K)}{(E)}$
1	0.0018	0.0018
2.5	0.0031	0.0012
5	0.0067	0.0013
7.5	0.011	0.0015
10	0.016	0.0016
25	0.038	0.0015
50	0.069	0.0014

mining the temperature coefficients. The values for K and Q_{10} are given in Table I. Fig. 5 shows the logarithms of the velocity constants, K, plotted against the reciprocals of the absolute temperature for each case. Under optimal conditions of pH, the points for the three temperatures are not far from a straight line, suggesting that a single fundamental reaction determines the rate of lysis by virolysin and that a similar—but not necessarily identical reaction—

controls the rate of lysis by autolysin over the range tested. The values of μ, the critical thermal increment, calculated from the van't Hoff-Arrhenius equation,

$$K_2 = K_1 e^{\mu/2} \frac{(T_2 - T_1)}{T_2 T_1},$$

range from 11,800 to 20,000.

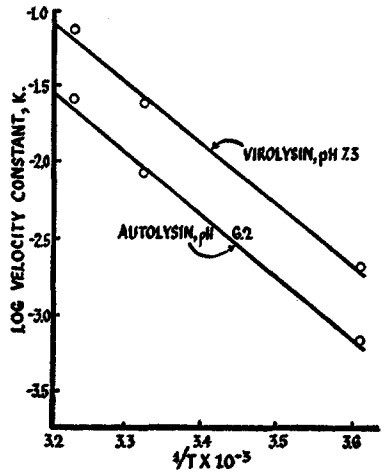

FIG. 5. Logarithms of velocity constants plotted against reciprocals of absolute temperature for lysis of *S. aureus* by virolysin and autolysin at their pH optima over the range 0–37°C.

TABLE I

Values of Velocity Constants and Temperature Coefficients for Lysis of Heat-Killed Staphylococci by Virolysin and Autolysin

Lysin	pH	$K \times 10^{-2}$, min.			Q_{10}	
		0°C.	28°C.	37°C.	0–28	28–37
Virolysin	7.3	0.288	3.0	5.32	4.3	1.9
Autolysin	6.2	0.047	0.77	2.03	6.8	2.9

III. Properties of the Lysins

A. pH Optima.—Differences in the pH optima of both crude and semi-purified lysins were shown by the following experiment: Lysis by autolysin and virolysin was determined over a wide pH range, both when the two agents were mixed together and when tested individually. Autolysin showed its maximum activity between pH 6.0 and 6.5 and virolysin between pH 7.0 and

7.5. The velocity constants for the lysis brought about by the mixture of the two lysins at each pH value (over the range pH 5.5 to 7.5) were found to equal the sum of the K values for each tested individually (Table II), indicating that the effects of mixing the two were additive, and that neither preparation contained any materials which might have affected the pH optimum of the other. Therefore, the two pH optima result from intrinsic differences between the two lysins.

B. Antigenic Relationships.—(*1*) *Antiserum to autolysin* inhibits lysis of killed K_1 cells by autolysin, but does not affect the action of virolysin. The antiserum was prepared by injection of rabbits with semipurified K_1 autolysin. The antiserum inhibited autolysin at a dilution of 1/50, but did not inhibit virolysin,

TABLE II

Lysis of Heat-Killed K_1 Cells by Autolysin, Virolysin, and Their Mixture at 37°C.

pH tested	Crude lysin preparation			
	Velocity constant, K/min., for lysis of K_1 cells by			
	Autolysin A	Virolysin B	Mixture M	Theoretical K, if M = A + B
5.5	0.0017	0.0017	0.0037	0.0034
6.5	0.0034	0.0028	0.0063	0.0062
7.5	0.0011	0.0040	0.0047	0.0051
8.5	0.0000	0.0020	0.0034	0.0020*

Active autolysin and virolysin mixed in equal parts at room temperature. Aliquots adjusted to various pH values with 1 N HCl or NaOH. Autolysin diluted 1:2 in boiled virolysin and virolysin diluted 1:2 in boiled autolysin for tests of individual lysin activity. Cell controls contained both inactive lysins (boiled) at the desired pH. Readings made at intervals by Klett turbidimetric method. Data were corrected for the per cent resistant cells.

* At pH 8.5, M varies somewhat above and below A + B from experiment to experiment.

even at 1/10. When autolysin and virolysin were mixed and treated with antiserum, the residual activity of the mixture equalled the activity of the virolysin alone, indicating that the virolysin preparation contained neither antiserum-sensitive nor antiserum-inactivating materials (Table III).

Table III shows that the antiserum also inhibits lysis-from-without by the combined action of autolysin and phage, but fails to inhibit lysis-from-without by virolysin and phage, indicating again that the antiserum is specific for autolysin.

The antiserum to K_1 autolysin has been found to inhibit the autolysin from *S. aureus* 145, but it has no effect on the virolysin 14(145) from this strain (table III).

(*2*) *Antiserum to Phage P_1*,—prepared by injection of rabbits with ultracentrifuged pellets containing phage, causes strong inactivation of P_{14} phage

but fails to inhibit either autolysin or virolysin at 1/200, a concentration which inactivated 10^8 phage particles per ml. in 20 minutes at 20°C.

These tests show that the two lysins are antigenically different from each other and that both are different from the phage P_1 (antigenically identical with P_{14}).

C. Effect of Chemical Inhibitors.—Both lysins are inhibited by the chemicals listed in Table IV. When cysteine is added to virolysin or autolysin which has been inhibited by compounds of heavy metals, activity is restored, suggesting that functional SH groups may be involved (Sumner and Somers, 1947).

TABLE III

Effect of Antiserum to K_1 Autolysin on Lysis by Autolysin and Virolysin Lysin Activity

Lysin	A Per cent lysis heat-killed K_1 cells by		B Per cent lysis heat-killed 145 cells by		C Per cent lysis phage-sensitized K_1 cells by	
	Untreated lysin	Neutralized lysin	Untreated lysin	Neutralized lysin	Untreated lysin	Neutralized lysin
K_1 autolysin.....	16	0	—	—	32	8
14(K_1) virolysin....	23	22	—	—	63	67
Mixture A + V....	43	27	—	—	—	—
145 autolysin.....	29	2	32	3	—	—
14(145) virolysin...	47	45	51	51	—	—

For neutralization, lysins incubated with 1/25 antiserum or normal serum, for 2 hours at 20°C. Residual lysin tested by adding 6×10^8 cells per ml., incubating 30 minutes at 37°C., and calculating per cent lysis by turbidimetric readings.

Reinvestigation of our earlier results with iodoacetate disclosed that whereas concentrations in the order of M/1000 were not inhibitory, greater amounts, in the order of M/100 were effective.

Quantitative differences were observed in concentrations of certain inhibitors required. The activity of crude virolysin was depressed by M/5000 $PbCl_2$; that of crude autolysin was hardly affected. With Na citrate, M/100 caused inhibition of crude autolysin but not of virolysin. These differences may have been due to impurities in the crude lysin preparations and to the influence of pH on the inhibition, since each test was carried out at the pH optimum of the lysin.

Na desoxycholate had no inhibitory action on the crude lysins, but it depressed the activity of their semipurified preparations.

D. Effect of Versene on Virolysin and Autolysin.—The addition of 0.02 per cent versene (disodium salt of ethylenediaminetetraacetic acid) to either lysin causes strong inhibition of lysis of heat-killed K_1 cells. Various metallic cations are able to reverse this inhibition: M/1000 Ca^{++}, Mn^{++}, Co^{++}, and Zn^{++}. Mg^{++} is effective only for virolysin at M/1000, and concentrations 20-fold greater failed to reverse inhibition of autolysin (Table V).

TABLE IV

Inhibitors of Virolysin and Autolysin Activity for Heat-Killed S. Aureus K_1 and M. Lysodeikticus

Inhibitor	Final concentration	Effect on virolysin Per cent inhibition of lysis	Effect on autolysin Per cent inhibition of lysis	
		S. aureus K_1	*S. aureus* K_1	*M. lysodeikticus*
$CuSO_4$	M/1000	83	27	13
"	M/200	—	100	100
Merthiolate	0.1 per cent	45	29	51
"	0.2 per cent	—	59	39
Iodoacetate	M/1000	21	3	0
"	M/100	93	61*	—
Formalin	0.1 per cent	93	82	17
"	0.4 per cent	—	100	70
$AgNO_3$	M/1000	97	95	100
$HgCl_2$	M/1000	100	29	23
Duponol WA	0.1 per cent	100	82	60
Na-citrate	M/100	7	56	26
" "	M/20	83	80	48
I_2 in KI	M/1000	100*	79*	—
$K_4Fe(CN)_6$	M/300	100*	100*	—
$PbCl_2$	M/5000	53*	15*	17*
"	M/2500	47*	50*	—
Na-desoxycholate	0.2 per cent	80*	86*	—

* Experiment conducted with semipurified lysin; otherwise, tests run with crude lysins.

Lysin and inhibitor were mixed with constant numbers of test cells, 8×10^8 cells per ml. in tryptose phosphate broth. Samples were incubated at 37°C. After a constant time, dependent upon the activity of the untreated lysin control, the number of bacteria lysed was estimated from the turbidimetric readings. The per cent inhibition of lysis was calculated from:

$$1.0 - \frac{\text{per cent lysis with inhibitor}}{\text{per cent lysis without inhibitor}} \times 100$$

The following agents failed to inhibit autolysin and virolysin: M/1000 semicarbazide, Na-azide, Na-arsenite, sulfanilic acid, ascorbic acid, Na-fluoride, thiourea, K-oxalate, Na-sulfite, Na-tetraborate; 0.0025 per cent proflavin; 0.1 per cent pectin, 0.001 per cent acriflavin; 0.1 per cent saponin; 20 per cent toluene, and chloroform.

E. Thermolability.—Crude virolysin and crude autolysin are completely inactivated by boiling for 5 minutes. Crude virolysin is unstable even at 37°C., whereas crude autolysin is not (Table VIA), possibly because of certain protective substances in crude autolysin preparations. These substances reduce the heat inactivation of crude virolysin at 37°C., when the two crude lysins are mixed together. These materials in the crude autolysin are at least partially removed by precipitation of the lysin with $(NH_4)_2SO_4$.

TABLE V

Versene Inhibition of Autolysin and Virolysin and Its Reversal by Divalent Cations

Sample contents	Virolysin, pH 7.3		Autolysin, pH 6.5
	Per cent lysis heat-killed K_1 after 60 min., 37°C.	Per cent lysis heat-killed K_1 after 60 min., 37°C.; overnight at 4°C.	Per cent lysis heat-killed *lysodeikticus* 180 min., 37°C.; and overnight at 4°C.
Lysin control L	55	41	33
Lysin, L, + versene 0.02 per cent, V	12	13	11
L + V + Mg^{++} M/50	30	12	6
L + V + Mg^{++} M/1000	31	12	19
L + V + Mn^{++} M/1000	40	49	43
L + V + Ca^{++} M/1000	45	43	40
L + V + Co^{++} M/1000	39	40	32
L + V + Zn^{++} M/1000	26	42	30

Aliquots of lysin, diluted in double strength tryptose phosphate broth, exposed to versene, inorganic salt, or equivalent volumes of distilled water. Cells added to 8×10^8 bacteria per ml.

Both semipurified lysins are thermolabile. The inactivation of semipurified virolysin at 37°C. and of semipurified autolysin at 37° and 45°C. is given in Tables VIB and VIII. While the semipurified autolysin may be more heat-resistant than the semipurified virolysin, this may be the result of incomplete removal of the non-specific protective substances in the crude autolysin.

F. Specificity: Spectrum of Bacteria Lysed by Autolysin and Virolysin.—*(1) S. aureus* K_1.—With both lysins, heat-killed logarithmic phase cells are more sensitive than heat-killed resting cells. Acetone treatment has often increased the sensitivity of heated resting cells to virolysin but not to autolysin.

(2) Heterologous S. aureus Strains.—Both lysins act on all heat-killed *aureus* strains (25) tested so far. The ratio of their relative activities, however, varies from strain to strain.

(3) Heterologous Micrococcus Species.—Heat-killed cells of the following species are resistant to both lysins: *M. tetragenes*, *M. roseus*, *M. citreus*, *M. agilis*, *Sarcina sp.* Heated cells of *M. lysodeikticus* are relatively resistant to virolysin but very susceptible to autolysin; the degree of susceptibility apparently depends on cell age and method of subsequent treatment (Table VII).

TABLE VI

Heat Inactivation of Crude and Semipurified Lysins at 37°C.

	pH at which sample was heated at 37°C. and tested	Time at 37°C.	Virolysin-autolysin mixture			Theoretical if $M_{2hrs.} = \frac{1}{2}V_{0hrs.} + A_{2hrs.}$
			Residual lysin *K* per min.			
		hrs.				
A. Crude lysins	6.0	0	0.008	0.007	0.020	
		2	0.002	0.005	0.013	0.013
	6.8	0	0.022	0.005	0.033	
		2	0.008	0.009	0.029	0.031
	7.6	0	0.016	0.002	0.020	
		2	0.009	0.004	0.017	0.020
B. Semipurified lysins	6.3	0	0.002	0.027		
		2	0.000	0.013		
	6.8	0	0.006	0.020		
		2	0.000	0.016		
	7.3	0	0.007	0.014		
		2	0.000	0.009		
	7.8	0	0.006	0.005		
		2	0.000	0.008		

In A, crude autolysin was diluted in boiled virolysin, virolysin diluted in boiled autolysin for tests of individual lysin heat lability. The active lysins were diluted in each other for tests of heat lability for mixture. Samples adjusted to desired pH with 1 N NaOH or HCl, heated for 2 hours, and residual lysin was determined by turbidimetric measurement of lysis of 8×10^8 bacteria per ml. of heated K_1 cells at 37°C. *K* per minute calculated from data corrected for 50 per cent lysis-resistant cells.

In B, same procedure used for semipurified lysins (the semipurified lysins were not prepared from crude lysins used in A). Mixture of the 2 semipurified lysins showed less heat protection of virolysin by autolysin.

TABLE VII

Effect of K_1 Autolysin, 14(K_1) Virolysin, and Their Mixture on Lysis of Acetone-Killed M. lysodeikticus at pH 6.2–8.0 at 4°C.

pH	Relative lysin activity per cent lysis			
	Experimental			Theoretical
	V	A	A + V	A + V
6.2	0	34	34	34
6.6	1	40	37	41
7.0	0	23	22	23
7.4	2	24	29	26

V = virolysin, A = autolysin, A + V = mixture.

Autolysin and virolysin were diluted one-half in each other or in boiled (inactive) lysin. Cells of acetone-extracted *M. lysodeikticus* were added to a final concentration of 8×10^8 bacteria per ml., after adjusting pH with NaOH or HCl. The per cent lysis was determined from turbidimetric readings after 2 days at 4°C. The concentrations of virolysin and autolysin used in the test were sufficient to cause extensive lysis of heat-killed K_1 cells.

Conceivably the strong activity of autolysin for *M. lysodeikticus* could be due to a contaminating lysin present in autolysin preparations but absent from virolysin preparations. This is not probable, because (1) autolysin preparations show the same pH optimum for K_1 and *M. lysodeikticus*, (2) the same chemical inhibitors prevent the lysis of both hosts by autolysin preparations, (3) heating

TABLE VIII

Inactivation at 45°C. of Autolysin Activity for Killed K_1 and M. lysodeikticus

Time heated at 45°C.	Residual activity, per cent lysis of 8 × 10^8 bacteria per ml. after 1 hr., 37°C., in samples heated and then tested at pH									
	5.8		6.3		6.8		7.3		7.8	
	Lys.	K_1	*Lys.*	K_1	*Lys.*	K_1	*Lys.*	K_1	*Lys.*	K_1
min.										
0	25	15	30	34	29	28	14	15	7	14
15	25	21	24	28	23	20	8	8	7	8
30	20	20	22	21	21	15	9	4	5	2
60	17	13	21	25	17	15	3	0	3	6

Logarithmic phase K_1 and *M. lysodeikticus* cells (*Lys.*) heat-killed for 60 and 30 minutes respectively just prior to test. Autolysin preparation: semipurified lysin at pH 6.5 and adjusted aliquots with 1 N NaOH and HCl. Heated at 45°C. and tested at same pH.

at 45°C. destroys the lytic activity in autolysin preparations at comparable rates for both hosts (Tables IV and VIII).

A second possible reason for the observed difference in activity could have been that virolysin preparations contained an inhibitor which prevented its action on *M. lysodeikticus*. This was not so, as indicated by testing mixtures of the two lysins over a pH range (Table VII). With *lysodeikticus* cells resistant to virolysin, the lysis in the mixture of the two lysins equalled that caused by autolysin alone. The results are best explained by assuming that the two lysins show different substrate specificities.

4. Heterologous Genera.—Simultaneous comparisons of both autolysin and virolysin have shown the following to be resistant: *Escherichia coli*, *Serratia marcescens*, *Bacillus mycoides*, *Bacillus subtilis*.

The following have been found to be resistant to virolysin and were not tested with autolysin: *Azotobacter chroococcum*, *Streptococcus species*, *Neisseria perflava*, *Propionibacterium jensenii*, *Corynebacterium xerose*, *Brucella melitensis*, *Listeria monocytogenes*, *Rhodospirillum rubrum*, *Caulobacter species*, *Streptomyces species*, *Mycobacterium tuberculosis*, *Cytophaga johnsonae*, *Anabaena cylindrica*.

Certain pseudomonads underwent slight but reproducible lysis with both lysins: *Pseudomonas fluorescens*, *Ps. aeruginosa*, *Ps. species*. It is uncertain whether this effect is caused by autolysin and virolysin, since no tests were made to rule out the possibility that a contaminating lysin could be responsible.

DISCUSSION

Both virolysin and autolysin produce lysis of cells which have been boiled for several hours, a procedure which destroys the intracellular enzymes of the bacterium. It is assumed, therefore, that no bacterial enzymes are involved in the action of either lysin and that the lysins act directly upon a heat-stable component of the cell. Both lysins have many of the properties associated with enzymes. Each is thermolabile, is destroyed by proteolytic enzymes, has a pH optimum, has substrate specificity, is inhibited by chemicals known to be "enzyme inhibitors," etc. With respect to the influence of temperature, lysis has the characteristics of a chemical reaction.

These findings suggest that cell lysis results from a chemical reaction involving a bacterial substrate and that this reaction is catalyzed by the enzyme virolysin or autolysin. The substrate may be a polysaccharide (Lieberman, 1956; Lieberman and Krueger, to be published) and probably forms the framework of the cell wall.

Since the lysins cannot act on cells unless they have been previously damaged by a "sensitizing" agent, it appears that a protective mechanism normally exists to prevent either lysin from acting on the substrate.

The lysin produced by the phage-infected cell is antigenically distinct from the lysin of the uninfected cell; this implies that the phage-synthesizing apparatus is not necessarily restricted to the use of preformed bacterial enzymes but rather can induce the appearance of an enzyme which is not present in detectable quantity in the normal cell.

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